C25

Adenovirus-mediated *in vivo* gene transfer and expression in normal rat liver

H. A. Jaffe[1], C. Danel[1], G. Longenecker[1], M. Metzger[1], Y. Setoguchi[1], M. A. Rosenfeld[1], T. W. Gant[2], S. S. Thorgeirsson[2], L. D. Stratford-Perricaudet[3], M. Perricaudet[3], A. Pavirani[4], J.-P. Lecocq[4] & R. G. Crystal[1]

Replication deficient, recombinant adenovirus (Ad) vectors do not require target cell replication for transfer and expression of exogenous genes and thus may be useful for *in vivo* gene therapy in hepatocytes. *In vitro*, primary cultures of rat hepatocytes infected with a recombinant Ad containing a human α1–antitrypsin cDNA (Ad–α1AT) synthesized and secreted human α1AT for 4 weeks. In rats, *in vivo* intraportal administration of a recombinant Ad containing the *E. coli lacZ* gene, was followed by expression of β-galactosidase in hepatocytes 3 days after infection. Intraportal infusion of Ad–α1AT produced detectable serum levels of human α1AT for 4 weeks. Thus, targeted gene expression has been achieved in the liver, albeit at low levels, suggesting that adenovirus vectors may be a useful means for *in vivo* gene therapy in liver disorders.

[1]*Pulmonary Branch, National Heart, Lung, and Blood Institute and* [2]*Laboratory of Experimental Carcinogenesis, National Cancer Institute, National Institutes of Health Bethesda, Maryland 20892, USA*
[3]*Institute Gustave Roussy, Centre National de la Recherche Scientifique Unité Associée 1301, 94805, Villejuif Cedex, France,* [4]*Transgene SA, 11 rue de Molsheim, 67082 Strasbourg, France*

Correspondence should be addressed to R.G.C.

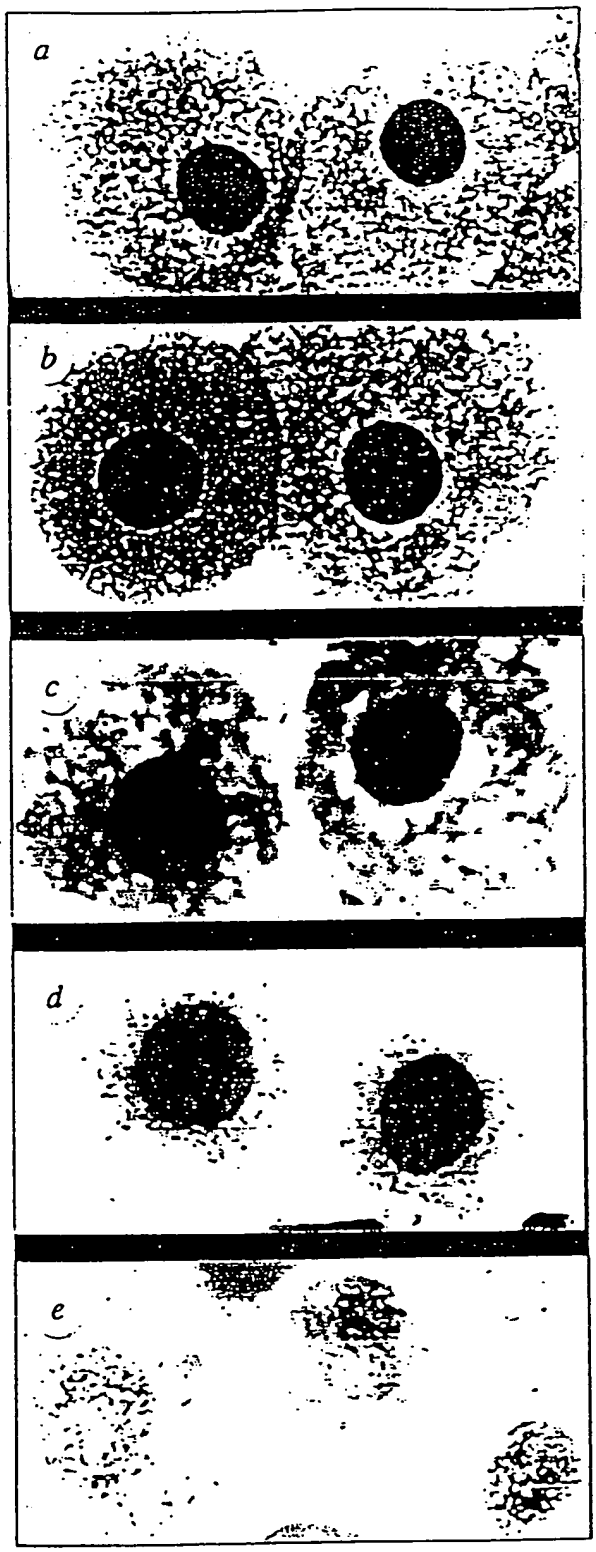
A significant challenge in the development of gene therapy for hereditary and acquired disorders in man is the expression of the therapeutic gene in the relevant cell population. The hepatocyte represents an example of this dilemma. Although the hepatocyte plays a central role in the pathogenesis of many human disorders, the low rate of cell proliferation has limited the use of retrovirus vectors, liposome-DNA complexes, and ligand-based DNA complexes for gene therapy, to models where hepatocyte replication can be enhanced, where the exogenous gene can be transferred to the hepatocyte *in vitro* and the modified cells reinfused, or where transient and/or relatively low level expression of the transferred gene may be sufficient to achieve the therapeutic goal[1–21].

To circumvent the requirement for ongoing hepatocyte replication for successful *in vivo* gene transfer, we studied adenovirus (Ad) vectors, which do not require host cell proliferation for expression of the gene to be transferred[22,23]. Recombinant, replication-deficient Ad vectors can accommodate large exogenous genes and have been used successfully in transferring human genes to the respiratory epithelium *in vivo*, cell targets that also replicate infrequently[24,25]. Hepatocytes, although rarely, can be targets for clinical adenovirus infection[26,27], and because the respiratory epithelium and the liver share the same embryologic origin[28] and genes can be transferred to the neonatal liver by recombinant Ad vectors[29], we hypothesized that the adult liver could be a target for *in vivo* Ad-mediated gene transfer. From a safety viewpoint, there are extensive epidemiologic data documenting that Ad infections are not associated with human malignancies and live adenovirus vaccines have been used safely in large human populations for more than two decades[26,30].

In the present study, the possibility of using the Ad to transfer human genes to hepatocytes *in vivo* was evaluated using the *E. coli lacZ* gene and a human α_1-antitrypsin (α1AT) cDNA as model genes. The expression of the *lacZ* gene, coding for β-galactosidase, serves as a model to identify cells in which there has been successful *in vivo* gene transfer. The expression of the human α AT cDNA serves as a model of the ability of the Ad vector to confer upon the hepatocyte the capacity to synthesize a typical liver-produced secretory glycoprotein, as directed by the transferred gene. It also provides a convenient protein for evaluation of chronicity of expression by following serum levels of the gene product[31].

Adenoviral transfer to cultured hepatocytes

Primary cultures of rat hepatocytes exposed to the recombinant Ad vectors demonstrated transfer and expression of the exogenous genes. The exposure of cultured hepatocytes to the *lacZ* containing Ad vector (Ad.RSVβgal; see Methodology) at a multiplicity of infection of 100 pfu per cell for 24 h resulted in expression of β-galactosidase in the majority (62%) of the hepatocytes with no associated change in the morphology of the cells (not shown). Exposure of the cultured hepatocytes to an Ad vector containing the human α1AT cDNA (Ad-α1AT)[24,32] resulted in expression of this cDNA and also caused no morphologic changes (Fig. 1*a*, *b*). *In situ* hybridization analysis of uninfected hepatocytes with a human α1AT antisense cRNA probe demonstrated no human α1AT mRNA transcripts (background level of grains), that is, the human α1AT antisense probe did not



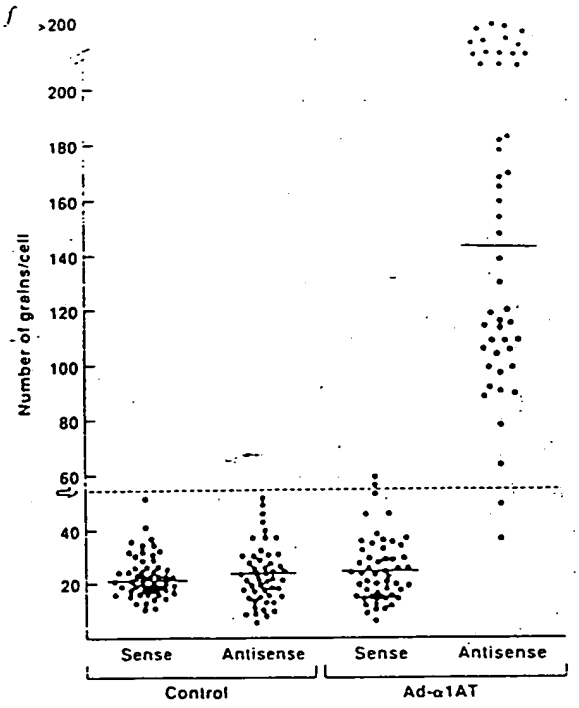


Fig. 1 Expression of human α1-antitrypsin (α1AT) mRNA in rat hepatocytes following adenovirus-mediated transfer of the human α1AT cDNA *in vitro*. Primary cultures of rat hepatocytes were infected *in vitro* with Ad-α1AT, a replication deficient, recombinant adenovirus (Ad) based on Ad type 5, containing a human α1AT cDNA driven by the Ad2 major late promoter. *In situ* hybridization evaluation of rat hepatocytes for the presence of human α1AT mRNA transcripts after 72 h (all original magnifications × 1000). *a*, Uninfected hepatocytes, Wright-Giemsa. *b*, Hepatocytes after infection with Ad-α1AT, Wright-Giemsa. *c*, Uninfected hepatocytes, [^{35}S]UTP-labelled antisense human α1AT cRNA probe. *d*, Ad-α1AT infected hepatocytes, antisense probe. *e*, As *d*, but evaluated with a control [^{35}S]UTP-labelled sense α1AT cRNA probe. *f*, Quantification of silver grains exposed over control and Ad-α1AT infected hepatocytes evaluated with the sense and antisense probes. Each symbol represents a randomly selected cell within the defined populations. The arrow and dashed line designate the number of silver grains 3 standard deviations above the mean number of grains exposed over controls: uninfected hepatocytes evaluated with the sense and antisense probes and Ad-α1AT infected hepatocytes evaluated with the sense probe.

recognize the endogenous rat hepatocyte α1AT mRNA transcripts (Fig. 1*c*). Three days after Ad-α$_1$AT infection, however, the same antisense probe clearly demonstrated human α1AT mRNA transcripts in rat hepatocytes (increased numbers of grains over the cells, Fig. 1*d*). The control sense probe demonstrated no transcripts (background level of grains, Fig. 1*e*). Quantitative assessment of the Ad-α1AT infected cultures with the antisense probe demonstrated that >95% of the viable cells had a higher number of autoradiographic grains than parallel cultures evaluated with the sense probe or uninfected hepatocytes evaluated with sense or antisense probes, suggesting that the α1AT cDNA was transferred to the majority of the cells *in vitro* (Fig. 1*f*). The viability of Ad-α1AT infected cultures was similar to uninfected or control Ad vector infected cultures.

Examination of the Ad-α1AT infected primary rat hepatocytes revealed that they were synthesizing and secreting human α1AT *de novo* (Fig. 2*a*). [^{35}S]methionine labelling for 24 h, and evaluation of the culture supernatants by immunoprecipitation with an anti-human α1AT antibody demonstrated secretion of human α1AT with the appropriate migration for mature, glycosylated α1AT molecules (relative molecular mass 52,000 [M_r

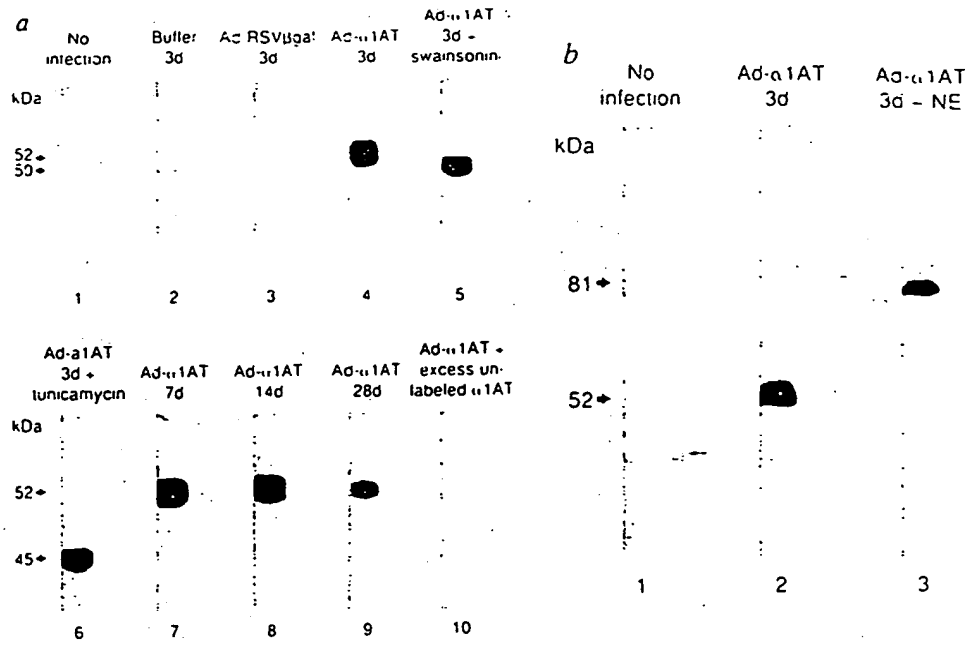


Fig. 2 Evaluation of *in vitro* Ad-α1AT infected rat hepatocytes for production of human α1AT and functional analysis of Ad-α1AT infected rat hepatocyte produced human α1AT by [^{35}S]methionine labelling, immunoprecipitation with an anti-human α1AT antibody, SDS-PAGE and autoradiography. *a*, Synthesis, secretion and characterization of human α1AT over time by Ad-α1AT infected rat hepatocytes. Lane 1, uninfected hepatocytes; lane 2, hepatocytes exposed to viral suspension buffer; lane 3, hepatocytes evaluated 3 days after infection with Ad.RSVβgal, a recombinant, replication deficient Ad containing the *E. coli lacZ* gene; lane 4, hepatocytes evaluated 3 days after infection with Ad-α1AT; lane 5, as lane 4, but with the addition of swainsonine; lane 6, as lane 4, but with the addition of tunicamycin; lane 7, hepatocytes evaluated 7 days after infection with Ad-α1AT; lane 8, hepatocytes evaluated 14 days after infection with Ad-α1AT; lane 9, hepatocytes evaluated 28 days after infection with Ad-α1AT; and lane 10, hepatocytes evaluated 28 days after infection with Ad-α1AT but with excess unlabelled human α1AT added to the supernatant prior to immunoprecipitation to block the antibody. Shown are the positions of migration of the 52K human α1AT, the partially glycosylated (the effect of swainsonine) 50K α1AT, and the completely non-glycosylated (the effect of tunicamycin) 45K α1AT. *b*, Capacity of human α1AT produced by Ad-α1AT infected rat hepatocytes to complex with neutrophil elastase (NE). Lane 1, uninfected hepatocytes; lane 2, hepatocytes evaluated 3 days after infection with Ad-α1AT; lane 3, as lane 2 but with the addition of human neutrophil elastase (500 nM final concentration) to the supernatant prior to immunoprecipitation. Shown are the positions of migration of the 52K human α1AT and the 81K complex of human α1AT and NE.

52K), compare lane 4 to no infection, virus suspension buffer and Ad. RSVβgal infection controls, lanes 1–3). The fact that the secreted α1AT has the apparent *Mr* of glycosylated a1AT is important because glycosylated a1AT has a half-life in the circulation 50-fold greater than that of non-glycosylated α1AT[33]. Further evidence that the newly synthesized human α1AT was glycosylated in a normal fashion came from comparison of α1AT when the Ad-α1AT infected hepatocytes were incubated in the presence of swainsonine (an inhibitor of carbohydrate side chain processing in the Golgi) or tunicamycin (an inhibitor of core glycosylation in the rough endoplasmic reticulum); the apparent M_r dropped to 50K (lane 5) and 45K (lane 6)), respectively[34,35]. Ad-α1AT infected hepatocytes continued to produce α1AT for at least 28 days (lanes 4, 7–9). The specificity of the antibody for human α1AT was demonstrated by adding excess unlabelled human α1AT to the immunoprecipitation reaction (lane 10). When neutrophil elastase (NE, the natural substrate of α1AT) was added to the supernatants of Ad-α1AT infected hepatocytes, the expected 81K α1AT-NE complexes[34] were observed (Fig. 2*b*).

Adenoviral transfer *in vivo*

In vivo gene transfer to the liver was first evaluated by introduction of Ad.RSVβgal or controls (virus suspension buffer, or control viruses Ad-dl312, Ad-α1AT, or Ad-CFTR (see Methodology)) into the liver via intraportal injection (Fig. 3). Rats observed for up to 90 days showed no adverse effects (altered survival, recovery time post-surgery, coat appearance or general activity level) compared to controls. Livers exposed to the Ad vectors *in vivo* revealed no gross or microscopic anatomical derangements when compared to the livers recovered from non-injected or virus suspension buffer injected animals. Livers recovered 3 days after infusion of an equivalent volume of virus suspension buffer or 10^{10} pfu Ad-α1AT revealed no evidence of *lacZ* gene expression (Fig. 3*a*, *b*). Likewise, infusion of 10^{10} pfu of Ad-dl312 or Ad-CFTR or no infusion at all resulted in no β-galactosidase activity (not shown).

In contrast, livers recovered from rats having received 10^{10} pfu intraportal Ad.RSVβgal 3 days previously showed expression of the exogenous gene in hepatocytes, as seen by the nuclear blue staining, characteristic of β-galactosidase activity as directed by the *lacZ* gene together with the SV40 nuclear localization signal. The distribution of positive cells was discontinuous: some areas showed randomly distributed single positive cells (Fig. 3*c*), other areas showed more closely spaced (Fig. 3*d-f*) or clumps (Fig. 3*f*, *g*) of blue staining hepatocytes. Some specimens had broad areas with no positive cells. Interestingly, no Kupffer cells, biliary tract cells or hepatic vascular endothelial cells expressed β-galactosidase activity. Examination of blindly selected samples of liver exposed to intraportal Ad.RSVβgal documented an average of 1.1 ± 0.2% (mean ± SEM; 661 of 59,839 total hepatocytes scored in a total of 2 livers) of hepatocytes expressing the *lacZ* gene. Animals receiving intraportal Ad.RSVβgal showed no evidence of β-galactosidase activity in heart, lung, brain, kidney, spleen, muscle or testis (not shown). Endogenous thyroid cytoplasmic β-galactosidase activity was uniformly present in Ad.RSVβgal and control animals and served as a positive control[36].

In contrast to direct intraportal administration of the Ad.RSVβgal vector, attempts at *in vivo* adenoviral gene transfer to the liver by intravenous injection of 10^{10} pfu Ad.RSVβgal ($n = 5$) or, as control, virus suspension buffer

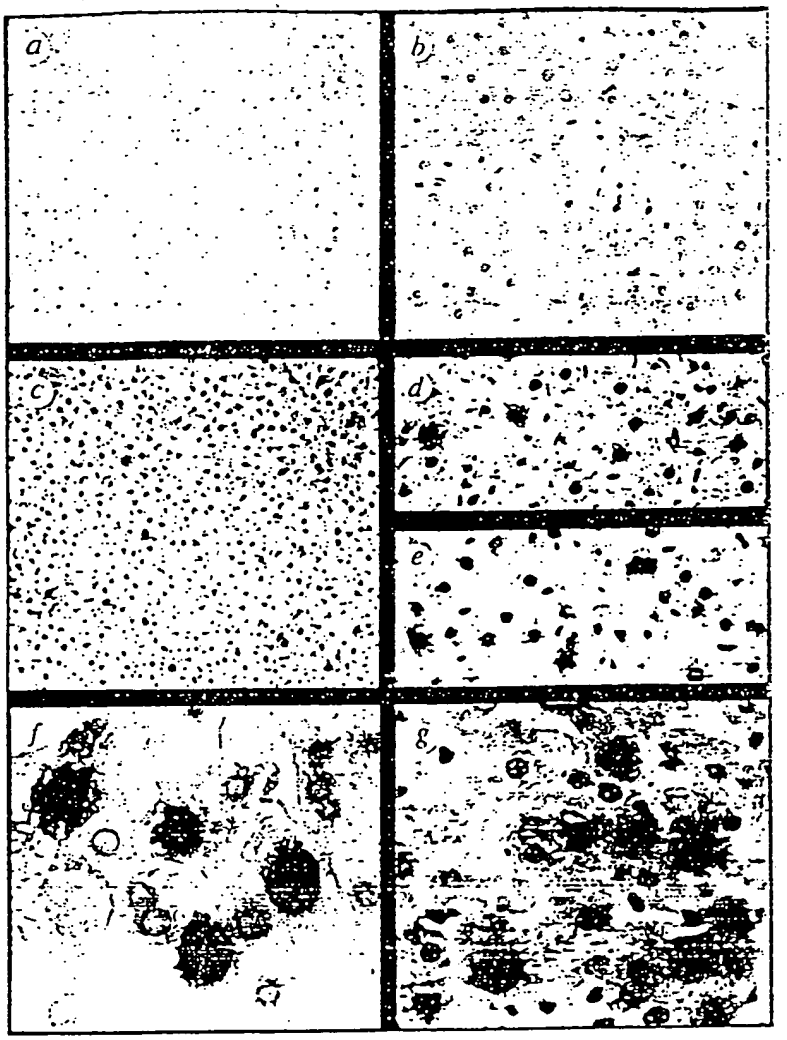


Fig. 3 *In vivo* transfer of the *E. coli lacZ* gene to rat hepatocytes. Three days after Ad.RSVβgal was infused into the portal vein, the liver was evaluated for the presence (blue staining) of the *lacZ* product β-galactosidase using X-gal stain. *a*, Liver section from a rat not receiving an intraportal infusion. X-gal stain, haematoxylin and eosin × 200. *b*, Liver section 3 days after intraportal infusion of Ad-α1AT. X-gal, haematoxylin and eosin × 400. *c*, Liver section 3 days after intraportal infusion of Ad.RSVβgal. X-gal, haematoxylin and eosin, × 200. *d*, and *e*, As *c* but × 400. *f*, Liver section 3 days after intraportal infusion of Ad.RSVβgal, X-gal and eosin, × 1,000. *g*, As *c*, but × 1,000.

($n = 3$) into the tail veins of adult rats were unsuccessful. Livers recovered 3 days after injection from either group did not show β-galactosidase activity (not shown).

As another approach to assessing the *in vivo* transfer of the *lacZ* gene to hepatocytes, we recovered hepatocytes by collagenase perfusion[37] of the liver 3 days after intraportal injection of Ad.RSVβgal. Staining with X-gal revealed a population of hepatocytes with nuclear blue staining consistent with the *in vivo* transfer of the *lacZ* gene (not shown). Aside from the expression of β-galactosidase activity, this population was in no other way distinguishable from hepatocytes recovered by the same methodology from uninfected rats or rats injected with Ad-α1AT, Ad-CFTR, Ad-dl312 or buffer, which stained negative.

In vivo transfer of α1-antitrypsin

Consistent with the observations obtained with Ad.RSVβgal, *in vivo* intraportal infusion of Ad-α1AT produced synthesis of the human α1AT in hepatocytes and the secretion of human α1AT into the circulation. No gross or microscopic anatomical derrangement of these livers was found compared to controls. Following intraportal administration of Ad-α1AT (10^{10} pfu) or controls (an equivalent volume of virus suspension buffer, or 10^{10} pfu of Ad-dl312, Ad.RSVβgal, or Ad-CFTR), hepatocytes were isolated at intervals 3–90 days thereafter and the production of α1AT analysed by [^{35}S]methionine labelling and immunoprecipitation. The *de novo* synthesis and secretion of human α1AT protein was documented by the immunoprecipitation of the expected 52K human ^{35}S-labelled α1AT from cultures of rat hepatocytes recovered by collagenase perfusion at 3, 7, 14, 28, 60 and 90 days after intraportal infusion of Ad-α1AT (not shown). No human α1AT was immunoprecipitated from similar hepatocyte supernatants from uninfected rats or other controls (other Ad vectors or viral suspension buffer). The immunoprecipitable signal eliminated by excess unlabelled human α1AT added to the immunoprecipitation reaction, confirmed that this was indeed human α1AT.

Expression of the *in vivo* transferred human α1AT cDNA was documented *in vivo* by quantification of serum levels of human α1AT with a human α1AT-specific ELISA (Fig. 4). Human α1AT in the circulation of rats having received intraportal Ad-α$_1$AT averaged 215–380 ng ml^{-1} for up to 28 days ($n = 25$, $p<0.005$ compared with controls over the same time period using the two-tailed Student's t-test; the normal level of endogenous rat α1AT in the circulation is 1.1 mg ml^{-1} (ref. 38)). A few 'anecdotal' animals demonstrated detectable human α1AT in the circulation for as long as 60 or 90 days. No human α1AT was found in serum samples from control rats ($n = 47$), including those receiving no intraportal infusion and those infused with control Ad vectors or viral suspension buffer. Survival was identical in all injected animal groups: sham, viral suspension buffer, control adenoviruses and Ad-α1AT.

Discussion

It is clear that replication-deficient Ad vectors can be used to transfer exogenous genes to hepatocytes *in vivo*, including genes coding for an intracellular functional protein (*E. coli* β-galactosidase) and a glycosylated, secreted functional protein (human α1AT). Our data support a new strategy for transfer and expression of exogenous genes into adult hepatocytes *in vivo* without prior perturbation of the predominant G_0 cell cycle state of the adult hepatocyte. This obviates the use of stimulants for hepatocytes such as hepatotoxins or resections, which have significant drawbacks for adaptation to human gene transfer. Several important topics are relevant to the potential usefulness of an Ad vector system for *in vivo* human gene transfer to the liver, including: 1) organ specificity; 2) extent of expression; 3) chronicity of expression and 4) safety.

Organ specificity to the liver was achieved by local administration. The Ad enters cells by interacting with cell surface receptors that recognize the fibre-penton structure of the virus capsid[38,39]. Following internalization, the virus is released from the virus receptor endosome and the virus moves to the nucleus[38,40,41]. The specificity for cell targets is likely a function of receptor density, Ad type and local immune surveillance. For targeting to the liver, the most direct approach is to infuse the Ad vector into the hepatic vascular supply such as the portal vein as used here. Importantly, this approach successfully targeted hepatocytes, as no Kupffer cells, biliary tract cells or hepatic vascular endothelial cells expressed the *lacZ* gene following infusion of Ad.RSVβgal. Moreover, the expression was liver-specific.

Adenovirus-mediated gene transfer for amelioration of hepatic enzyme deficiency has been attempted in other model systems. In a mouse model of ornithine transcarbamylase deficiency (OTC) (a gene normally expressed at a relatively low level in liver) systemic administration of an adenovirus vector containing a rat OTC cDNA to newborn mice resulted in some animals with enhanced levels of hepatic enzyme activity and some exhibiting phenotypic correction[29]. Systemic administration of adenovirus vectors to newborn mice, however, results in the exogenous gene expression in multiple organs including lung, liver, intestine, heart and skeletal muscle[29,42]. Moreover, in transgenic mouse studies where OTC transgenes were expressed predominantly in the intestine, the phenotypic defect was also corrected[43,44] raising the question of the contribution of enzyme activity from sites other than the liver. In the current study, adult rats were given an Ad vector directly to the liver via intraportal injection and expression was limited to the liver.

The relatively low levels of α1AT achieved (Fig. 4) in comparison with a level that would be required for therapy (>0.8 mg ml^{-1}) must be considered with the knowledge that, unlike OTC, the α1AT gene is expressed in hepatocytes at a high level. Two other fundamental differences exist between the current study and the newborn mouse model. First, adult rats are not necessarily comparable to newborn mice in terms of susceptibility to *in vivo* gene transfer. Second, we determined the serum concentration of α1AT whereas in the newborn mouse model only the activity of the (intra-mitochondrial) OTC was assessed. Despite these differences, a comparison of the studies suggests that direct intraportal administration to adult yields local expression at a level less than that of a highly expressed protein (α1AT), whereas systemic administration to neonates results in expression in multiple organs of sufficient activity to produce phenotypic correction for a relatively low level expressed protein (OTC). These concepts will have to be taken into account in designing gene transfer strategies to specific organs.

The extent of expression of the *lacZ* gene within the liver following intraportal Ad.RSVβgal infusion was patchy, with approximately 1% of hepatocytes demonstrating β-galactosidase activity 3 days after infusion. However, >60% of hepatocytes in primary cultures expressed β-galactosidase when infected *in vitro* at a multiplicity of infection of 100 pfu per cell with the same vector, suggesting that the extent of expression *in vivo* may be determined by the amount of functional

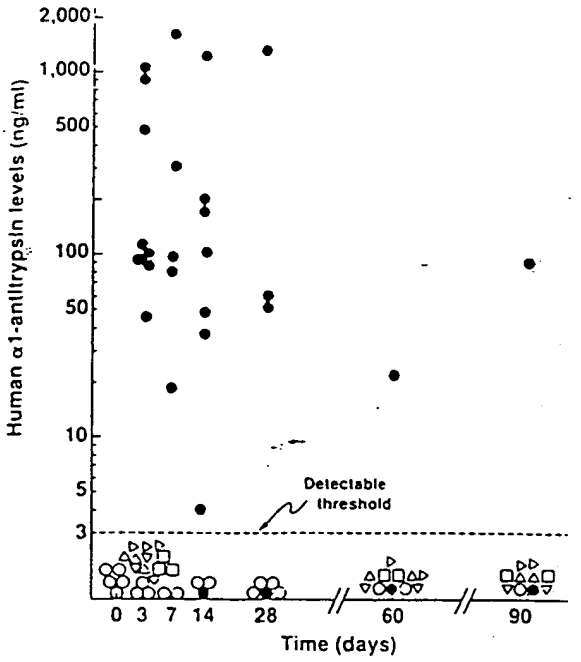


Fig. 4 Levels of human α1AT in the circulation of rats after intraportal administration of Ad-α1AT. Ad-α1AT (10^{10} pfu) or Ad control vectors (10^{10} pfu each) or buffer was infused into the portal vein of anesthetized rats. Each symbol represents the mean of quadruplicate determinations from an individual rat. Shown are data for Ad-α1AT infected animals •, and a variety of controls: o, Rats receiving no intraportal infusion; ▽, rats receiving intraportal virus suspension buffer; △, Ad-dl312; □, Ad-CFTR; ▷, Ad.RSVβgal. No α1AT was detected in the preparations of Ad-α1AT used for infection.

vector reaching each hepatocyte. Since the rat liver contains approximately 10^9 hepatocytes[45], infusion of 10^{10} pfu will permit, at most, 10 pfu per cell. Moreover, "pfu" for the Ad vectors are defined on a cell line (293, transformed human embryonal kidney cells)[46] which provides E1 function in *trans* to the replication-deficient Ad vectors. This *in vitro* titering method may not reflect the infectivity of rat hepatocytes *in vivo*. The patchy *in vivo* expression may also be affected by the distribution of the perfusate bolus after injection into the portal vein. Because the intraportal adenovirus strategy does not require prior hepatic damage to enhance hepatocyte replication[11,12,14,15] or partial hepatectomy to harvest hepatocytes for *in vitro* gene transfer and reimplantation[18,21], it avoids the natural morbidity and mortality of these procedures. Furthermore, optimization of the *in vivo* adenovirus delivery system (for example, by higher dosages or repeated administration) may offer significant increases in exogenous gene expression and/or the duration of expression of the transferred gene.

The expression achievable using the present strategy would not be sufficient to treat human α1AT deficiency but might possibly be sufficient to provide enough recombinant molecules (alone or with conventional therapy) to treat a variety of genetic disorders, including the factor VIII and factor IX haemophilias; deficiency disorders of the urea cycle, disorders of amino acid metabolism, carbohydrate metabolism, or porphyria related disorders[1,47-49]. The extent of expression seen here

may allow therapy for disorders such as LDL receptor mutations, should the receptor systems be sufficiently potent[4,20,21,47,48], or in females with X-linked disorders where the pattern of X-inactivation has left them with suboptimal levels of gene expression[3,47,49].

In reference to chronicity of expression, the available evidence suggests that the Ad genes likely function in an extra-chromosomal fashion — little is integrated in the genome[22]. While this has advantages as a vector system in minimizing insertional mutagenesis, the chronicity of infection may be limited by the rate of cell turnover. The expression for at least 4 weeks of the human α1AT cDNA following *in vivo* transfer to the liver is consistent with the slow turnover of adult hepatocytes *in vivo*[21]. Alternatively, the exogenous gene may persist in the hepatocyte but expression may decline as has been observed for genes transferred to cells *in vitro* by recombinant retrovirus vectors and then reimplanted[52]. This may be obviated, however, by repeated dosages.

Finally, the safety of replication deficient recombinant adenovirus vectors *in vivo* in humans is unknown and further study will be needed to resolve this issue. There is evidence to suggest however, that safe use may be possible. At no time interval after *in vivo* transfer with Ad vectors was there gross or microscopic evidence of hepatic damage, regenerative or degenerative changes, inflammatory responses, or evidence of neoplastic changes. Importantly, human Ad infection is not associated with malignancy. The Ad vectors are constructed to be replication deficient; while it is conceivable that exogenous wild type Ad could complement the deleted region to propagate the recombinant virus, Ad hepatic infection is a rare clinical event in humans[26,27], suggesting this may not be an important safety issue. Finally, oral Ad vaccines have been safely used in humans for decades with an excellent safety record[30].

Methodology

Hepatocyte culture. Hepatocytes were isolated from Sprague Dawley rats (250–350 g) by hepatic perfusion with 140 U ml^{-1} collagenase (Type IV, Sigma), 10 mM HEPES, pH 7.4 in Williams' media E (GIBCO)[47]. Recovered cells were allowed to adhere for 4 h on tissue culture plates (Becton Dickinson, Lincoln Park, New Jersey) in Dulbecco's modified Eagle medium (DMEM) mixed 1:1 with Waymouth's medium (both from GIBCO) including dexamethasone 20 ng ml^{-1} (Sigma), insulin 5 μg ml^{-1}, transferrin 5 μg ml^{-1}, selenous acid 5 ng ml^{-1} (ITS, Collaborative Research, Bedford, Massachusetts), gentamicin 10 μg ml^{-1}, 10 mM HEPES pH 7.4, and 10% heat inactivated fetal calf serum (Hyclone Laboratories, Logan, Utah). Nonadherent cells were removed by washing twice with Ca^{++}/Mg^{++}-free Hank's balanced salt solution (HBSS). Histologic evaluation demonstrated >98% of the cells had the typical appearance of hepatocytes. Cultures were continued in maintenance media that differed from plating media by the deletion of fetal calf serum and the inclusion of bovine serum albumin 1.25 mg ml^{-1} and linoleic acid 5.35 μg ml^{-1} (ITS+, Collaborative Research).

Adenovirus vectors. Adenoviruses were prepared, purified and titered as described[24,25,47,53]. Ad-α1AT is an Ad5 E1a, E3 deletion based vector with the Ad2 major late promoter and the human α1AT cDNA[24,25]. Ad.RSVβgal is an Ad5 E1, E3 deleted replication deficient, recombinant adenovirus identical to Ad-CFTR (see ref. 25) except that in place of the Ad2 major late promoter and human cystic fibrosis transmembrane conductance regulator (CFTR) cDNA, there is the Rous sarcoma virus long terminal repeat, the SV40 nuclear localization signal, and the *E. coli lacZ* gene[42,54,55]. Ad-dl312 is an Ad5 E1a deletion mutant without an exogenous gene[25]. Ad-CFTR is an Ad5 based, E1, E3 deletion vector otherwise similar to Ad-α1AT, but with the human (CFTR) cDNA[25] instead of the α1AT cDNA.

In vitro gene transfer to hepatocytes. After the change to maintenance media, hepatocyte cultures were infected with Ad-α1AT or Ad.RSVβgal at a multiplicity of infection of 100 pfu per cell. Control, replicate cultures were exposed to equivalent volumes of virus suspension buffer (10 mM Tris-HCl, 1 mM MgCl2, 10% glycerol, pH 7.4) or media alone. For the *in situ* analyses, hepatocytes were recovered after 72 h by exposure to trypsin-ethylenediaminetetraacetic acid (2 min, 37 °C: Trypsin/Versene, Biofluids, Rockville, Maryland), gentle scraping and cytocentrifugation. Human α1AT mRNA transcripts were evaluated in cytocentrifuge preparations with [^{35}S]UTP-labelled sense and antisense cRNA probes of similar specific activity and concentration (1.2×10^8 dpm μl^{-1}) prepared from a human α1AT cDNA insert in pGEM-3Z (Promega). After hybridization, preparations were exposed to autoradiography film for 4 days and counterstained with Wright-Giemsa[56,57]. For evaluation of human α1AT synthesis, secretion and function the hepatocytes were isolated and infected as above. After 3, 7, 14, or 28 days the cells were incubated (24 h, 37 °C) with [^{35}S]methionine (250 μCi ml^{-1} (>1000 Ci mmol^{-1}; New England Nuclear)). To demonstrate that the human α1AT was glycosylated in a normal fashion, parallel cultures of Ad-α1AT infected hepatocytes were labelled with [^{35}S]methionine after the addition of tunicamycin (10 μg ml^{-1}, an inhibitor of core glycosylation) or swainsonine (5 μg ml^{-1}, an inhibitor of distal glycosylation processing (both from Boehringer Mannheim Biochemicals)). Supernatants were evaluated by immunoprecipitation with a rabbit anti-human α1AT antibody (Boehringer Mannheim), SDS-polyacrylamide gel electrophoresis and autoradiography. The function of the human α1AT produced was demonstrated by addition of human neutrophil elastase (Elastin Products, Owensville, Maryland) prior to immunoprecipitation[24].

In vivo gene transfer to liver. Sprague Dawley rats (250–350 g) were anaesthetized with a mixture of ketamine HCl and xylazine. The peritoneal cavity was incised and the portal vein isolated and injected with 10^{10} pfu of Ad.RSVβgal or Ad-α1AT. The abdomen was closed and the rats allowed to recover. After 3 days, some animals were sacrificed, bled, and the liver fixed *in situ* by perfusion with PBS (4 °C) followed by 4% paraformaldehyde (Fisher Scientific, Fair Lawn, New Jersey) in PBS (4 °C). Livers were then minced into 2 mm fragments, and evaluated for β-galactosidase activity by incubation in X-gal staining solution (5 mM K4Fe(CN)6, 5 mM K3Fe(CN)6, (both from Sigma), 2 mM MgCl2 (Mallinckrodt), and 200 μg ml^{-1} X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, Boehringer Mannheim))[56]. After staining, tissues were embedded in paraffin, sectioned, adhered to glass slides, and counterstained with hematoxylin and eosin or eosin alone. Controls included no injections or intraportal injection of Ad-dl312, Ad-CFTR or an equivalent volume of virus suspension buffer. The remaining control animals and those receiving intraportal Ad-α1AT or Ad.RSVβgal were sacrificed at 3, 7, 14, 28, 60 and 90 days after injection, bled, hepatocytes isolated (described above), and the *de novo* synthesis and secretion of human α1AT analysed by [^{35}S]methionine labelling and immunoprecipitation[24]. The *in vivo* expression of the Ad-α1AT transferred α1AT cDNA was analysed in the serum samples with a human α1AT-specific ELISA with a sensitivity of ≥3 ng ml^{-1}.

Received 30 March; accepted 25 June 1992.

Acknowledgements

We thank P. Lemarchand and C. Jonas for helping in this study and A. Avent for editorial assistance.

1. Horwich, A.L. *Curr. Topics Micro. Immunol.* 168, 185–200 (1991).
2. Friedmann, T. *Science* 244, 1275–1281 (1989).
3. Wilson, J.M. and Chowdhury, J.R. *Molec. biol. Med.* 7, 223–232 (1990).
4. Miller, A.D. *Blood* 76, 271–278 (1990).
5. Ledley, F.D. *J. Inher. metab. Dis.* 13, 597–616 (1990).
6. Nicolas, C., Le Pape, A., Soriano, P., Fargette, F. & Jonet, M-F. *Proc. natn. Acad. Sci. U.S.A.* 80, 1056–1072 (1983).
7. Soriano, P. *et al. Proc. natn. Acad. Sci. U.S.A.* 80, 7126–7131 (1983).
8. Kaneda, Y., Iwai, K. & Uchida, T. *Science* 243, 375–378 (1989).
9. Kaneda, Y., Iwai, K. & Uchida, T. *J. biol. Chem.* 264, 12126–12129 (1989).
10. Kato, K., Nakanishi, M., Kaneda, Y., Uchida, T. & Okada, Y. *J. biol. Chem.* 266, 3361–3364 (1991).
11. Wu, C. H., Wilson, J. M. & Wu, G. Y. *J. biol. Chem.* 264, 16985–16987 (1989).
12. Wu, G.Y. *et al. J. biol. Chem.* 266, 14338–14342 (1991).
13. Wilson, J. M. *et al. J. biol. Chem.* 267 963–967 (1992).
14. Hatzoglou, M. *et al. J. biol. Chem.* 265, 17285–17293 (1990).
15. Kaleko, M., Garcia, J. V. & Miller, A. D. *Hum. Gene Ther.* 2, 27–32 (1991).
16. Demetriou, A.A. *et al. Science* 233, 1190–1192 (1986).
17. Ponder, K.P. *et al. Proc. natn. Acad. Sci. U.S.A.* 88, 1217–1221 (1991).
18. Kay, M. A. *et al. Proc. natn. Acad. Sci. U.S.A.* 89, 89–93 (1992).
19. Inagaki, M. & Ogawa, K. *Cell Struct. Funct.* 16, 283–288 (1991).
20. Wilson, J.M. *et al. Proc. natn. Acad. Sci. U.S.A.* 87, 8437–8441 (1990).
21. Chowdhury, J.R. *et al. Science* 254, 1802–1805 (1991).
22. Berkner, K.L. *Biotechniques* 6, 616–628 (1988).
23. Horwich, M.S. *Virology* 2nd edn(eds Fields, B.N. & Knipe, D.M.) 1679–1721 (Raven Press, New York, 1990).
24. Rosenfeld, M.A. *et al. Science* 252, 431–434 (1991).
25. Rosenfeld, M.A. *et al. Cell* 68, 143–155 (1992).
26. Straus, S.E. in *The Adenoviruses* (ed Ginsberg, H. S.) 451–496 (Plenum Press, New York & London 1984).
27. Griffiths, P.D., Ellis, D.S. & Zukerman, A. J. *Brit. Med. Bull.* 46, 512–532 (1990).
28. Allan, F.D. *Essentials of Human Embryology* (ed Allan, F. D.) 93–99 (Oxford University Press, New York, 1960).
29. Stratford-Perricaudet, L.D., Levrero, M., Chasse, J-F., Perricaudet, M. & Briand, P. *Hum. Gene Ther.* 1, 241–256 (1990).
30. Chanock, R.M., Ludwig, W., Heubner, R.J., Cate, T.R. & Chu, L-W. *JAMA* 195, 445–452 (1966).
31. Garver, Jr., R.I., Chytil, A., Courtney, M. & Crystal, R.G. *Science* 237, 762–764 (1987).
32. Gilardi, P., Courtney, M., Pavirani, A. & Perricaudet, M. *FEBS Lett.* 267, 60–62 (1990).
33. Casolaro, M.A. *et al. J. appl. Physiol.* 63, 2015–2023 (1987).
34. Elbein, A.D. *Ann. Rev. Biochem.* 56, 497–534 (1987).
35. Curiel, D.T., Chytil, A., Courtney, M. & Crystal, R.G. *J. biol. Chem.* 264, 10477–10486 (1989).
36. Pearson, B., Wolf, P.L. & Vazquez, J. *Lab. Invest.* 12, 1249–1259 (1963).
37. Seglen, P.O. *J. Toxicol. Environ. Health* 5, 551–560 (1979).
38. French, T. in *The Cunical Chemistry of Laboratory Animals* 1st edn(eds Loeb, W. F. & Quimby, F. W.) 213. (Pergamon Press, New York, 1989).
39. Lonberg-Holm, K. & Philipson, L. *J. Virol.* 4, 323–338 (1969).
40. Philipson, L., Lonberg-Holm, K. & Pettersson, U. *J. Virol.* 2, 1064–1075 (1968).
41. Pastan, I., Seth, P., FitzGerald, D. & Willingham, M. in *Concepts in Viral Pathogenesis II* (eds Notkins, A.L. & Oldstone, M.B.A.) 141–146 (Springer-Verlag, New York, 1986).
42. Stratford-Perricaudet, L.D., Makeh, I., Perricaudet, M. & Briand, P. *J. clin. Invest.* 90, 626–630 (1992).
43. Jones, S.N. et al. *J. biol. Chem.* 265 14664–14690 (1990).
44. Shimada, T. *et al. FEBS Lett.* 279 196–200 (1991).
45. Seglen, P.O. *Exptl. Cell. Res.* 82, 391–398 (1973).
46. Graham, F.L., Smiley, J., Russe, W.C. & Nairn, R. *J. gen. Virol.* 36, 59–74 (1977).
47. Venar, G.A., Lawn, R.M., Tuddenham, E.G.D. & Wood, W.I. in *The Metabolic Basis of Inherited Disease* 6th edn(eds Scriver, C.R., Beaudet, A.L., Sly, W.S. & Valle, D.) 2107–2122, 2155–2182. (McGraw-Hill, New York, 1989).
48. Brusilow, S.W. & Horwich, A.L. in *The Metabolic Basis of Inherited Disease* 6th edn(eds Scriver, C. R., Beaudet, A.L., Sly, W.S. & Valle, D.) 1395–491, 495–774, 1305–1406 (McGraw-Hill, New York, 1989).
49. Goldstein, J.L. & Brown, M.S. in *The Metabolic Basis of Inherited Disease* 6th edn(eds Scriver, C. R., Beaudet, A.L., Sly, W.S. & Valle, D.) 1215–1250 (McGraw-Hill, New York, 1989).
50. Am, P.H. *et al. New Engl. J. Med.* 322, 1652–1655 (1990).
51. Brues, A.M. & Marble, B.B. *J. exp. Med.* 65, 15–27 (1937).
52. Palmer, T.D., Rosman, G.J., Osborne, W. R. A. & Miller, A.D. *Proc. natn. Acad. Sci. U.S.A.* 88, 1330–1334 (1991).
53. Graham, F.L. & Van Der Eb, A.J. *Virology* 52, 456–467 (1973).
54. Yamamoto, T., de Crombrugghe, B., & Pastan, I. *Cell* 22, 787–797 (1980).
55. Kalderon, D., Roberts, B.L., Richardson, W.D. & Smith, A.E. *Cell* 39, 499–509 (1984).
56. Price, J., Turner, D., & Cepko C. *Proc. natn. Acad. Sci. U.S.A.* 84, 156–160 (1987).
57. Jones, N. & Shenk, T. *Cell* 17, 683–689 (1979).
58. Harper, M.E., Marselle, L.M., Gallo, R.C. & Wong-Staal, F. *Proc. natn. Acad. Sci. U.S.A.* 83, 772–776 (1986).
59. Bernaudin, J-F. *et al. J. Immunol.* 140, 3822–3829 (1988).
60. Dannenberg, Jr., A.M. & Suga, M. in *Methods for Studying Mononuclear Phagocytes* (eds Adams, D.O., Edelson P.J. & Koren, H.S.) 375–395 (Academic Press, New York, 1981).